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Forms of p185HER-2/neu in Breast Cancer

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*Gi. McCall*  
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## **(5)Introduction**

HER-2/neu encodes a transmembrane tyrosine kinase that functions as a potent oncogene and its over expression predicts poor prognosis and possibly response to adjuvant endocrine and chemotherapy (1-8). The soluble HER-2/neu extracellular domain (ECD) is proteolytically released from breast carcinoma cells in culture (9-11) and is elevated in sera of patients with metastatic breast cancer (7,11- 17). Patients with primary breast cancer (stage I-III) have lower levels of ECD in their sera than do those with metastatic disease (7,11,14,17). Release of soluble HER-2/neu may be accelerated in advanced stage disease, or levels may reflect tumor bulk. However, a recent study on soluble HER-2/neu in the blood of 211 patients with node positive disease found no correlation between elevated serum HER-2/neu and tumor size (16). Proteolytic release of the ECD is expected to leave an activated membrane-associated cytoplasmic fragment, since engineered removal of the extracellular domain enhances oncogenic potency of HER-2/neu (18-22) and several other receptor tyrosine kinases (12).

The overall purpose of the research project was to determine the signaling activity and prognostic significance of the truncated, variant HER-2/neu products in breast cancer. In the first year of this study, the scope of the research was to further characterize and determine the levels in breast cancer tissue of HER-2/neu product variants and to relate their levels to stage of the disease and patient outcome.

## **(6) Body:**

The focus during the current budget year was on characterizations of the N-terminally truncated HER-2/neu cytoplasmic domain in cultured cells and breast cancer tissue. Before we conducted extensive prognostic studies in breast cancer tissue, we had to step back and do more characterizations in cultured cells to ensure that we were looking at an activated kinase that was actually the tail of the shed HER-2/neu ECD and was a product generated in intact cells.

To summarize progress, we identified and characterized, for the first time, a truncated HER-2/neu protein, of about 95 kDa, that is missing the ectodomain (Fig. 1 and 2; Appendix A). Identity with the cytoplasmic domain of p185HER-2/neu was concluded because p95 reacted with two different antibodies, one against the kinase domain (Ceiba-Geigy) and a second against the C-terminal autophosphorylation site. Since p185 but not p95 reacted with antibody against the extracellular domain, we concluded that p95 was missing regions of the extracellular domain. P95 was an active kinase since when isolated by immunoprecipitation, it had autophosphorylation activity in the absence of p185 (Fig 1). p95 had matching phosphopeptides with p185HER-2/neu (Fig 2) which further established identify as a HER-2/neu product. The truncated cytoplasmic HER-2/neu product of about 95 kDa was in breast carcinoma cell lines but not in nontumorigenic breast epithelial cells (Fig. 3). Further our evidence supports that p95HER-2/neu is the cytoplasmic tail left when the ectodomain is shed indicated by its association with the membrane fraction (Fig 5 and 8) and on the correlation in their amount in different cells and under different treatment conditions (Figs 7 and 8).

Although not a stated aim or goal of our proposed study, we considered it important to generate evidence that p95 was actually the cytoplasmic tail of the shed ECD and not an unrelated product generated by artifactual degradation or as the product of an alternative transcript. Since the proteolysis of p185 into the truncated forms in cultured cells is limited, and since p95 contains only a fraction of methionines and cysteine as the full length receptor, our

attempts at pulse chase studies to show a precursor product relationship between p185 and ECD or p95 have been unsuccessful. Another strategy was developed to show a relationship between the ECD and p95 and to demonstrate that p95 was a proteolytic product generated in intact cells rather than an in vitro artifact. We analyzed the effects of several protease inhibitors and other effectors on the production of ECD and p95HER-2/neu in cultured, intact cells. Initially we examined the effects of the tumor promoter, TPA and chloroquine, an agent that alters pH and inhibits lysosomal enzymes. Fig. 7 and 8 illustrate that a combination of TPA and chloroquine stimulated ECD and amount of p95 in cells. Pupa et al. (11) first reported that leupeptin and EDTA, only when combined, extinguished production of ECD. We therefore examine the effects of these on coordinated production of p95 and ECD from BT474 cells and found that production of both truncated products were inhibited (see appendix, Fig. 4). Most recently, we tested the effects of the potent metalloprotease inhibitor, TAPI (provided by Immunex). We found that both p95 and ECD were inhibited in a dose related manner with an IC50 of about 1  $\mu$ M with this inhibitor. From these studies, we concluded that p95 correlates with ECD production, and that they are inhibited with the potent metalloprotease inhibitor, TAPI.

Importantly, p95HER-2/neu was in human breast cancer tissue, in some cases, at levels of 100% the amount of the full-length p185HER-2/neu (Fig 6, and see manuscript in the appendix). Our progress involved characterizations of HER-2/neu encoded protein products in 20 human tissues samples of primary intraductal adenocarcinoma of the breast at different stages of the disease. The overall amount of HER-2/neu expression was determined in membrane fractions of homogenized tissue using a kit obtained from Ciba Geigy. To determine the different variant forms of HER-2/neu, the samples (10  $\mu$ g protein) were resolved on small gels and analyzed by Western blot analysis. We used three different antibodies to assess the levels of each form, to verify that the bands under investigation by Western blotting were specific for HER-2/neu encoded proteins, and to identify regions that were contained or deleted from the truncated forms. Using antibodies against epitopes in the extracellular domain, we identified p185 full length receptor. Using antibodies against the C-terminus of the receptor we identified the full length p185 and a truncated form of 95kDa that migrated with the truncated cytoplasmic domain characterized in tissue culture. An additional antibody against the cytoplasmic domain of HER-2/neu provided by Ciba-Geigy also reacted with p185 as well as p95. The truncated p95 was from 5%-100% of the level of p185 in 20 different tissue samples. This result provided evidence for the presence of a truncated form of HER-2/neu in human breast cancer and suggested the possibility of dividing samples into subgroups based on level of p95 to discriminate patient subsets and test for association with disease outcome and survival time.

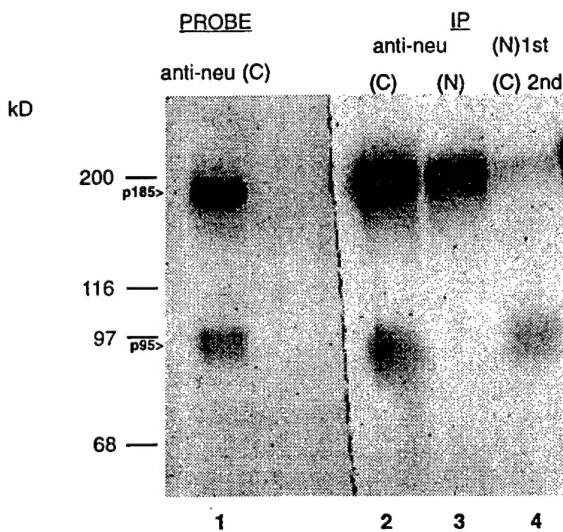
Progress was made on characterizing the signaling activity of the truncated cytoplasmic domain p95HER-2/neu based on its kinase activity and tyrosine phosphorylation in cells in culture and in breast cancer tissue samples. The kinase activity was measured in vitro immune complex kinase reactions, and the incorporation of phosphate into the protein was used to assess its kinase activity. The kinase activity of the truncated form, p95, was assessed in samples that had been cleared of p185HER-2/neu to confirm that p95 had its own kinase activity and was not a substrate of p185 (Fig.1). From these results we concluded that p95 was active in signal transduction since it had tyrosine kinase activity. To further assess whether p95 was an active signaling molecule, we tested for reactivity with antibodies specific for phosphotyrosine since tyrosine phosphorylation is a hallmark of activated receptor tyrosine kinase. Truncated p95 in breast cancer tissue and in breast carcinoma cells was found to be tyrosine phosphorylated

(Fig.5,6), however the extent of tyrosine phosphorylation was highly variable in the different tissue.

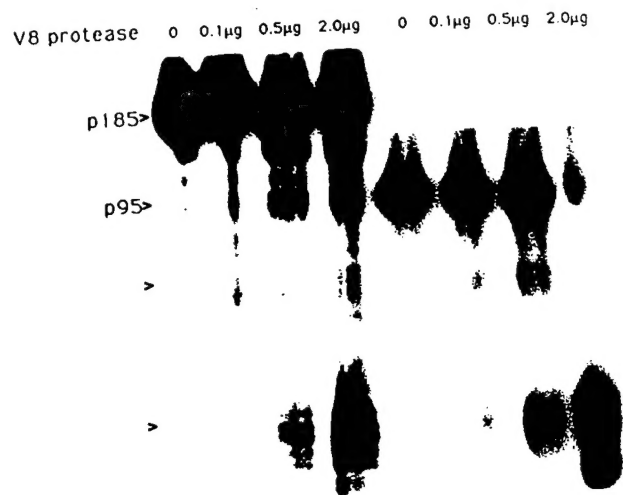
Our progress has generally fit into the broad context of the proposed purpose and scope of the original research plan, but with greater focus on the truncated p95CDHER-2/neu and characterization of its structure and function. This has included a diminution in efforts toward characterizing the ligand-binding domain variant, LBD-100 discussed in the original application. This decision was based on absence of clear cut function of the ligand binding domain in signaling and in overall activity of HER-2/neu in malignant progression whereas p95CD is known to be an active kinase and to have enhanced oncogenic potency based on numerous previous studies. Now that we have evidence that p95 is an active kinase, is the cytoplasmic tail of the shed ECD, we will now redirect our focus to studying breast tumor tissue. In addition, we will conduct further studies to evaluate the significance of p95 to signaling. Further, we will work toward our original plans to show the signaling activity of truncated HER-2/neu products by association with critical cytoplasmic signaling proteins initially by coimmunoprecipitation for example with phospholipase C. The additional goal in the second year will be to determine the levels of p95 relative to p185HER-2/neu in several breast cancer tissue and to relate the results to levels of soluble HER-2/neu and to disease stage. This will be used to assess the significance of p95 in disease progression and the relationship of p95 to soluble HER-2/neu.



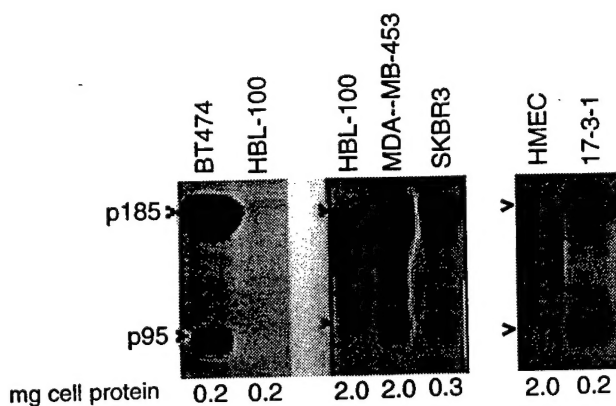
## Illustrations/Diagrams



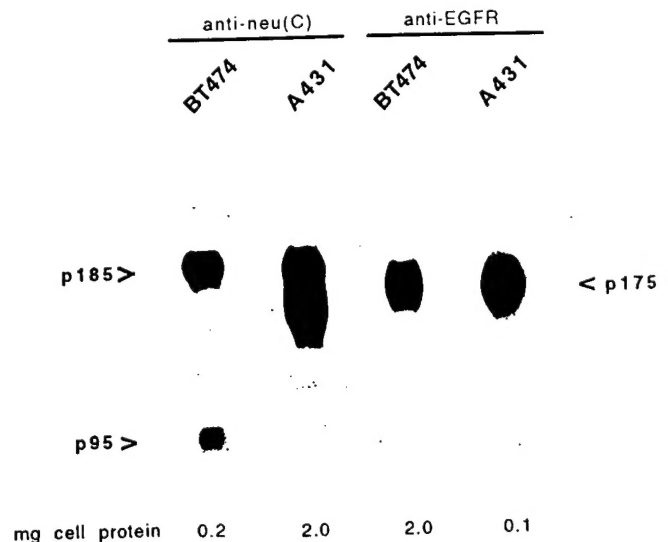
**Fig. 1. N-terminally truncated cytoplasmic domain of HER-2/neu with kinase activity is in transfected 17-3-1 cells.** Cell extracts were Western blotted with anti-neu(C) (lane 1) or immunoprecipitated with anti-neu(C) (lanes 2,4) or with monoclonal antibody against the extracellular domain, anti-neu(N) (lane 3), or an aliquot was depleted of p185HER-2/neu by extracting twice with anti-neu(N) and then was immunoprecipitated with anti-neu(C) (lane 4). The immune complexes were incubated in a kinase reaction containing ((-<sup>32</sup>P) ATP and analyzed by SDS-PAGE and autoradiography.



**Fig. 2. Partial proteolytic cleavage maps indicate matching phosphopeptides of autophosphorylated p185 and p95 HER-2/neu.** Excised gel pieces containing phosphorylated p185 and p95 from transfected 17-3-1 cells were loaded in a second gradient gel. Proteins in gel pieces were digested with V.8 protease and separated by electrophoresis.



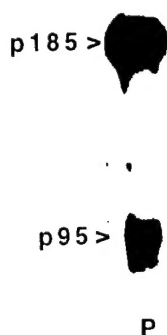
**Fig. 3 Human breast carcinoma cell lines but not nontumorigenic breast epithelial cells contain p95HER-2/neu.** To normalize for amount of HER-2/neu expression, varied amounts of cell lysates from the breast carcinoma cell lines, BT474, MDA-MB-453, SKBR3, and the nontumorigenic breast epithelium cells line, HBL-100 and normal human breast epithelial cells (HMEC) were all immunoprecipitated with anti-neu(C) and phosphorylated in the immune complex.



**Fig. 4 Truncated EGF receptor tyrosine kinase is not in BT474 breast carcinoma cells.** Different amounts of cell extract from BT474, or the human epidermoid carcinoma cell line, A431, were immunoprecipitated with anti-neu(C) or with polyclonal antipeptide antisera against the C-terminal autophosphorylation site of the EGF receptor (EGFR) and autophosphorylated in the immune complex.

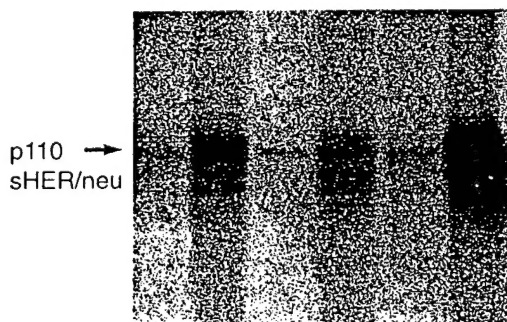


IP: anti-neu(C)  
probe: anti-PTyr

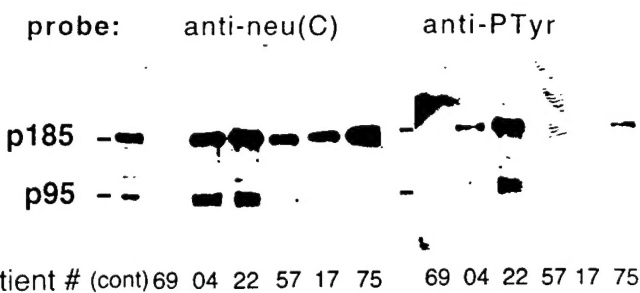


**Fig. 5. Tyrosine phosphorylated p95CD localized in the membrane fraction of BT474 breast carcinoma cells.** Particulate (P) and soluble (S) fractions were prepared by homogenization and centrifugation at 100,000 x g. The particulate fraction and an equivalent proportion of the soluble fraction were immunoprecipitated with anti-neu (C) and Western blotted with monoclonal anti-phosphotyrosine antibody.

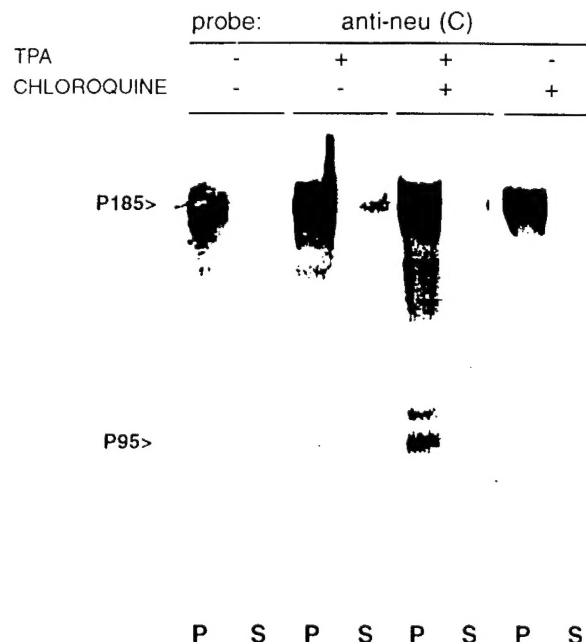
	IP: anti-neu (N)					
TPA	+	+	-	-	+	+
Chlorquine	-	-	+	+	+	+
Time (hr)	6	20	6	20	6	20



**Fig. 7. TPA and chlorquine enhance p110 soluble HER-2/neu in the extracellular medium.** BT474 cells, labeled with <sup>35</sup>S-methionine for 6hrs, were washed and treated for 6 hrs. or for 20 hrs with vehicle, or with 500 nM phorbol 12-myristate 13-acetate (TPA), or with 50  $\mu$ M chlorquine, or with TPA plus chlorquine. The conditioned medium was collected, concentrated and immunoprecipitated with monoclonal anti-neu(N).



**Fig. 6 P95HER-2/neu accumulates and is tyrosine phosphorylated in some primary human breast cancer tissue.** Six tissue samples, from primary human intraductal adenocarcinoma of the breast with #75, #17, and #04 at stage 2; #22 at stage 3, #57 at stage 4 and #69 at unknown stage were minced, homogenized, and fractionated by differential centrifugation into a particulate membrane-containing fraction and 10  $\mu$ g protein were resolved in minigels and immunoblotted first with monoclonal antibodies against phosphotyrosine (anti-PTyr), stripped and reprobed with anti-neu(C).



**Fig. 8. TPA and chlorquine enhance p95HER-2/neu in the membrane fraction.** BT474 cells were treated for 20 hrs with control vehicle, with 500 nM TPA, with 50  $\mu$ M chlorquine, or with TPA plus chlorquine. The cells were homogenized and fractionated by differential centrifugation into particulate and soluble fractions, and then western blotted using anti-neu(C) as probe.

## **(7) Conclusions:**

The truncated kinase domain of p185HER-2/neu that we discovered and characterized has important and direct significance to treatment and diagnosis of breast cancer. The truncated kinase domain, p95, is likely to play a role in malignant progression because it is a form of HER-2/neu with enhanced oncogenic potency. Importantly, it was present in breast cancer tissue where it could impact prognosis and the development of antibody based therapies aimed at blocking the action of HER-2/neu at the surface of breast cancer cells. If breast cancer tissue contains an abundance of p95CD that is active in signaling, then attempts at antibody therapy based on reactivity with extracellular regions of the receptor will fail.

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## **(9) Appendices**

Publications: Enclosed is a manuscript under review.

### Meeting Abstracts:

Doherty, J., Lin, Y.J., Christianson, T.A., Holmes, R., Keenan, E.J., and Clinton, G.M. Truncated HER-2/neu cytoplasmic domain with kinase activity in cultured cells and in human breast cancer. Abstract #1157 pp173. Proceedings of the AACR, Volume 38, March 1997.

Clinton, G.M., Christianson, T.A., Doherty, J., Holmes, R., Keenan, E., and Lin, J.J. Truncated HER-2/neu cytoplasmic domain with kinase activity corresponds to shed ECD in breast cancer. for "Era of Hope" The Department of Defense Breast Cancer Research Meeting. Oct. 31st, 1997.

Personnel Receiving pay from this effort:  
Dr. Gail M. Clinton, Principal Investigator  
Russell Moser, Research Assistant.

## **Truncated HER-2/neu kinase in breast carcinoma cells.**

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Edward J. Keenan<sup>2</sup>, and Gail M. Clinton<sup>1</sup>\*

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*keywords:* Truncated HER-2/neu (c-erbB-2) protein, tyrosine kinase, breast cancer

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### **Abstract**

The HER-2/neu extracellular domain (ECD) is in cancer patient's serum suggesting the truncated kinase will remain in cells. We describe p95HER-2/neu kinase in breast cancer cells which is likely the cytoplasmic tail generated by limited proteolysis of p185HER-2/neu, since its ECD was missing, it fractionated with membranes, was repressed by treatment of intact cells but not cell extracts with protease inhibitors, and correlated with ECD release. P95 was in 4 of 12 cancer tissues at 60-100% of p185HER-2/neu, a level that should significantly contribute to HER-2/neu kinase expression. P95 kinase may have prognostic significance and identify tumors with HER-2/neu-specific protease activity.

### **1. Introduction**

HER-2/neu (erbB-2) encodes a transmembrane tyrosine kinase, functions as a potent oncogene [1-3], and predicts poor prognosis when over expressed in human breast cancer [4-8]. The extracellular domain (ECD) of the HER-2/neu product is released from the surface of breast carcinoma cells by regulated proteolysis [9-11] and is elevated in sera of patients with breast [11-15], ovarian [16], and prostate [17-18] cancer. Patients with early stage breast and prostate cancer apparently have lower levels of ECD [11,12,15,17,18] which may reflect tumor mass and release of soluble HER-2/neu may be accelerated in some advanced stage disease.

Proteolytic release of the ECD is expected to leave a truncated HER-2/neu kinase domain in the cells. Several lines of evidence demonstrate that a kinase domain, missing its ECD, has constitutive signaling activity and enhanced oncogenic potency based on engineered deletion of the extracellular domain of HER-2/neu as well as several other receptor tyrosine kinases [19-23].

Here we describe a tyrosine phosphorylated, C-terminal fragment of HER-2/neu receptor, p95, that has kinase activity, is generated by proteolysis in intact cells, and correlates with release of soluble HER-2/neu from cultured breast carcinoma cells. P95HER-2/neu was found at relatively high levels in a subset of breast cancer tissue where it would not be detected in conventional clinical assays.

## **2. Materials and methods.**

### *2.1 Cells*

Cell lines were obtained from the American Type Culture Collection (Rockville, MD) except the 3T3 cells transfected with HER-2/neu cDNA, 17-3-1, were provided by Applied bioTechnology, Inc. (Cambridge, MA) and the human mammary epithelial cells (HMEC) cultured from tissue obtained from reduction mammoplasty were provided by Dr. Gary Shipley of Portland, Oregon. BT-474 and HMEC cells were cultured in RPMI medium supplemented with 10% FBS and 10µg/ml insulin and the antibiotic gentamicin at 0.5%. All other cell lines were grown in Dulbecco's Modified Eagles's Medium (DMEM). 17-3-1 cells were cultured in DMEM containing 0.4 mg/ml geneticin (G418, GIBCO-BRL).

### *2.2 Antibodies.*

Antipeptide antibody against the C-terminus of p185HER-2/neu, anti-neu(C), was previously described [24]. Monoclonal antibody against the extracellular domain of HER-2/neu was prepared as described [25] and was provided by Applied bioTechnology Inc. Monoclonal anti-phosphotyrosine antibody (p3300) was purchased from Sigma.

### *2.3 Cell fractionation.*

Cultured cells were washed and incubated on ice for 10 min in 10mM Tris pH 7.4, 10mM NaCl, 2mM MnCl<sub>2</sub> (TNM buffer) with protease inhibitors including 5mM EDTA, 5µg/ml leupeptin, 1% aprotinin, 1mM

phenylmethylsulfonylfluoride (PMSF). Suspended cells were Dounce homogenized, clarified by centrifugation (1500xg, 2 min) and the supernatant was fractionated into a particulate and soluble fraction by centrifugation at 340,000 x g for 1 hr. For fractionation of tissue, about 0.1 gm of primary human breast carcinoma, that was fresh-frozen and stored at -70°C, was minced on dry ice, suspended in TEDG buffer (50 mM Tris, 1.5 mM EDTA pH 7.5 with 0.5mM dithiothreitol, 10% glycerol and protease inhibitors, and homogenized using a Brinkman polytron. Clarified homogenates (1500 x g for 10 min) were centrifuged for 20 min at 40,000 x g at 4°C and the pellet containing the membranes was solubilized in modified RIPA buffer (M-RIPA) [24]. Samples of 10 µg of protein, determined by the BioRad protein assay, were subjected to Western blot analysis as described below.

#### *2.4 HER-2/neu tissue extract ELISA.*

Aliquots of breast cancer tissue prepared as described above, were assayed for protein content and the membrane-enriched extract was assayed using the Triton Diagnostics c-erbB-2 Tissue Extract EIA kit (Ciba Corning) according to manufacturer's instructions. The HER-2/neu units/mg protein in the specimens were calculated from a calibration curve generated by plotting the HER-2/neu concentration of the calibration standards versus the absorbance obtained from the EIA .

#### *2.5 Immunoprecipitation and western blotting.*

Cell lysates in M-RIPA and protease inhibitors were immunoprecipitated and resolved by SDS-PAGE as described [9,24]. Following SDS-PAGE of immunoprecipitates or cell lysates, proteins were electroblotted onto Bio-Blot nitrocellulose (Costar), reacted with primary and secondary antibodies as previously described [9,24], and developed with enhanced chemiluminescent reagent (ECL) (Amersham) according to manufacturer's instructions.

#### *2.6 Immune complex kinase assays.*

Freshly prepared cell lysates in MTG lysis buffer (20mM HEPES pH 8.0, 150 mM NaCl, 1% TritonX-100, 10% glycerol, and protease inhibitors ) were immunoprecipitated and the immune complex was washed twice



with MTG buffer and incubated 10 min on ice in a kinase reaction mixture containing 20 mM HEPES pH 8.0, 2 mM dithiothreitol, 25  $\mu$ M vanadate, 0.5% Nonidet P-40, 10 mM  $\text{MnCl}_2$ , 1  $\mu$ M ATP, and 15  $\mu$ Ci ( $\gamma$ - $^{32}\text{P}$ )ATP (New England Nuclear). The immune complex was washed 3 times with M-RIPA and released proteins were analyzed by SDS-PAGE.

### 3. Results

#### 3.1 Identification of N-terminally truncated HER-2/neu protein with kinase activity.

3T3 cells transfected with HER-2/neu cDNA (17-3-1 cells) release soluble ECD by proteolytic processing of p185HER-2/neu [10, and Lin and Clinton, unpublished observations]. To test for the presence of a truncated cytoplasmic domain, extracts were immunoblotted with antibodies against the C-terminus of the HER-2/neu product [anti-neu(C)] [24], which detected only two proteins, the full length p185HER-2/neu and a protein of about 95 kDa (Fig. 1, lane 1). The truncated product formed by *in vitro* deletion of HER-2/neu ECD sequence was of comparable size of 90-100kDa [12,13]. Extracts were immunoprecipitated with anti-neu(C) and only two proteins, a 95kDa protein and p185HER-2/neu, were phosphorylated in the immune complex with ( $\gamma$ - $^{32}\text{P}$ )ATP (Fig. 1, lane 2). A monoclonal antibody specific for the N-terminal region of p185HER-2/neu [anti-neu(N)] did not immunoprecipitate p95 showing that ECD epitopes were missing (lane 3). To examine whether p95 had self-phosphorylating activity or was a substrate of the full length receptor, p185 was depleted from the cell lysate with anti-neu (N). P95 self-phosphorylated in the immune complex kinase assay with greatly diminished p185 (Fig. 1 lane 4). Therefore, p95 appeared to correspond to an N-terminally truncated kinase domain encoded by HER-2/neu.

#### 3.2 P95HER-2/neu is in human breast carcinoma cells.

The breast carcinoma cell line, BT474, known to release soluble ECD [9] also contained only two autophosphorylated HER-2/neu products, p185 and p95, that were elevated compared to the nontumorigenic breast epithelial cell line HBL-100 (Fig. 2). To determine whether p95 was in proportion to levels of p185, cell extracts of HBL-100, human mammary epithelial cells, (HMEC), and three breast carcinoma cell lines were adjusted to

normalize for HER-2/neu expression [26] and proteins were phosphorylated with ( $\gamma^{32}\text{P}$ ) ATP. P95 was detected in the low (MDA-MB-453) and high (BT474 and SKBR3) HER-2/neu expressing breast carcinoma cells, but not in the HBL-100 nor HMEC cells, despite a robust signal from the HER-2/neu receptor which migrated as a slightly smaller protein in the breast epithelial cells (Fig. 2).

### *3.3 P95 correlates with ECD release and is inhibited by treatment of intact cells with protease inhibitors.*

P95 immunoprecipitated from BT474 cells with anti-neu(C), fractionated with p185 in the particulate membrane fraction and was tyrosine phosphorylated (Fig. 3). If p95 is the cytoplasmic tail, it should correlate with amount of soluble ECD. We previously found that phorbol 12-myristate 13-acetate (TPA) in combination with chloroquine, a drug that targets lysosomal and endosomal vesicles, markedly stimulate soluble HER-2/neu release from cells (Lin, Moser, and Clinton, in preparation). BT474 cells treated with TPA and chloroquine had seven to ten fold enhanced extracellular p110HER-2/neu and intracellular p95 (Fig. 4 A and B). (P110 and p95 from control cells were detectable upon longer exposures of the blot). HER-2/neu ECD production was reported to be blocked in cells treated with EDTA and the protease inhibitor leupeptin [11]. While treatment of BT474 cells with either EDTA or leupeptin alone had little or no effect on either p95 or the ECD (data not shown) intact cells stimulated with TPA and chloroquine that were exposed to EDTA combined with leupeptin had depressed amounts of both p95 and extracellular p110HER-2/neu (Fig. 4 A and B).

### *3.4 P95HER-2/neu is in primary human breast cancer.*

Twelve specimens of adenocarcinoma of the breast were selected for a range of HER-2/neu expression based on tissue immunoassay (Ciba Corning) to investigate the possible presence of p95. P95 was prominent in four samples based on reactivity with anti-neu(C) and comigration with p95 from the control 17-3-1 cells. An additional polyclonal antibody against the cytoplasmic domain of HER-2/neu product (Ciba Corning) also detected p95 and p185 in these tissue samples (data not shown). For samples #60 and #53, EIA value and amount of p185 relative to the control cells were low, yet p95 was present at an equivalent level to p185. Two additional samples, #04 and #22, had high EIA, p185, and p95 (about 60% of p185) compared to HER-2/neu products in the control

sample (Fig .5). Samples # 69 and 39 had little or no detectable HER-2/neu product in agreement with the EIA values of less than 100 Units/mg (Fig. 5). Five samples ( #57, 17, 75, 40, and 58) had a range of p185 and EIA values from low to high, yet displayed little or no p95.

## Discussion

Here we describe a HER-2/neu product, p95, that has a truncated N-terminus and contains the C-terminal autophosphorylation domain based on reactivity with domain-specific antibodies in western blots and immunoprecipitations. P95 is an active kinase since it self-phosphorylated without p185 and it was tyrosine phosphorylated in vivo.

Our results support the conclusion that p95HER-2/neu is generated, in intact cultured cells, by limited proteolysis and is the cytoplasmic tail of soluble HER-2/neu ECD. Cells extracted with protease inhibitors had, only two cytoplasmic HER-2/neu proteins, p95 and p185, with no indication of smaller degradation products. P95 was associated with the membrane fraction as would be expected following release of the ECD. P95 and HER-2/neu ECD levels were suppressed at the same time in cells treated with the protease inhibitor leupeptin combined with EDTA, which inhibits metalloproteases. On the other hand, p95 levels were not reduced when leupeptin and EDTA as well as several additional protease inhibitors were added during cell extraction and fractionation. Pulse chase experiments further show that p95 is a post-translational product (Lin, Moser, and Clinton, manuscript in preparation). Simultaneous augmentation of p95 and ECD by TPA and chloroquine treatment further demonstrates they are generated through the same mechanism. Truncated HER-2/neu of the same size, 95kDa, was found in several different cell lines and in breast cancer tissue suggesting that the same processing occurs in each case. Future studies aimed at testing cancer tissue and serum from the same patients will evaluate whether serum ECD correlates with tissue p95 in vivo.

The amount of p95HER-2/neu in different cells and in tumors varied and was not directly proportional to the levels of p185. There was no p95 signal in the nontumorigenic cells, HBL-100 or in HMEC even in large amounts of cell extracts when the high molecular weight receptor gave a robust signal. Also, p95 was not detected in some breast cancer tissues that had high amounts of p185. On the other hand, a relatively high level of p95 and low proportion of p185 were in sample #53 and #60. Pupa et al. [27] reported that production of HER-2/neu ECD

varies in cells and is not in proportion to levels of p185. These findings argue in favor of regulated formation of truncated HER-2/neu proteins rather than constitutive processing of a constant proportion of p185. According to this model, the levels of p95 and serum ECD would not only depend on tumor size and HER-2/neu expression, but some tumors could produce enhanced amounts of truncated HER-2/neu proteins.

Oncogenic signaling by HER-2/neu is known to depend on its kinase activity [2,3,11,12]. Since p95HER-2/neu had kinase activity and was 60-100% of p185 in some samples, it should significantly increase the kinase signal. By analogy to results with engineered deletions of the ECD from the HER-2/neu product [19-22], p95 is expected to emit an enhanced kinase signal. The *in vitro* tyrosine kinase activity of p95 in BT474 cells, estimated by autophosphorylation levels compared to western blotting signal, appeared several fold higher than that of p185 (compare BT474 cells in Fig. 2 and Fig. 4). Taken together these results suggest that p95 will elevate the kinase signal thereby promoting more aggressive tumor growth. This model needs to be tested in studies that correlate p95HER-2/neu with stage of disease and with patient outcome.

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#### Figure Legends

Fig. 1. N-terminally truncated HER-2/neu product with kinase activity. 25  $\mu$ g protein from 17-3-1 cells were western blotted with anti-neu(C) diluted 1:10,000 (lane 1) or immunoprecipitated (400  $\mu$ g protein) with anti-neu(C) (lanes 2,4) or with monoclonal antibody against the extracellular domain, anti-neu(N) (lane 3), or depleted of p185HER-2/neu by extracting twice with anti-neu(N) and then immunoprecipitated with anti-neu(C) (lane 4). The immune complexes were phosphorylated with ( $\gamma$ - $^{32}$ P) ATP and analyzed by SDS-PAGE and autoradiography.

Fig. 2. Human breast carcinoma cell lines contain p95HER-2/neu. Indicated amounts of cell lysates from BT474, HBL-100, MDA-MB-453, SKBR3, HMEC, and 17-3-1 cells were immunoprecipitated with anti-neu(C) and phosphorylated.

Fig. 3. Tyrosine phosphorylated p95HER-2/neu localized with membranes from BT474 cells. 200  $\mu$ g of the particulate (P) fraction and an equivalent proportion of the soluble fraction (S) prepared as described in **methods** were immunoprecipitated with anti-neu(C) and western blotted with anti-phosphotyrosine antibody.

Fig. 4. P95HER-2/neu correlates with ECD and is suppressed in intact cells by protease inhibitors. BT474 cells were treated for 24 hrs with control vehicle, with 500nM of the phorbol ester TPA and 50  $\mu$ M chloroquine, or with TPA, chloroquine, 5mM EDTA and 50  $\mu$ g/ml leupeptin. In A, 20  $\mu$ g cell proteins extracted in MRIPA with protease inhibitors including EDTA and leupeptin, and in B, 5ml of serum free conditioned medium from the treated cells were analyzed by western blotting. The results are representative of three replicate experiments.

Fig. 5. P95HER-2/neu is in a subset of breast cancer tissue. 12 specimens of human intraductal adenocarcinoma of the breast were fractionated and analyzed for EIA values and 10  $\mu$ g were subjected to western blotting with anti-neu(C). The control lane contained 10  $\mu$ g protein from transfected 3T3 cells, 17-3-1. EIA values were: <100 Units for #60, 589 U for #40, 458 U for #58, 302 U for #38, 200 U for #53, <100 U for #39. <100 U for #69, 2000 U for #04, 10,000 U for #22, 2000 U for #57, 220 U for #17, 674 U for #75.



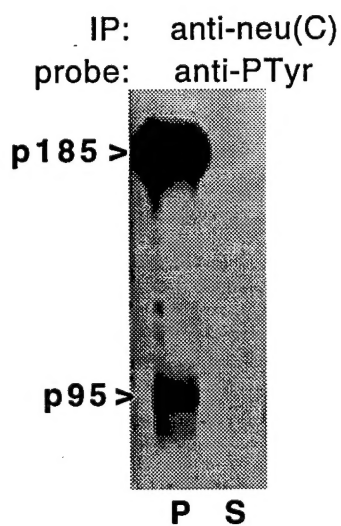
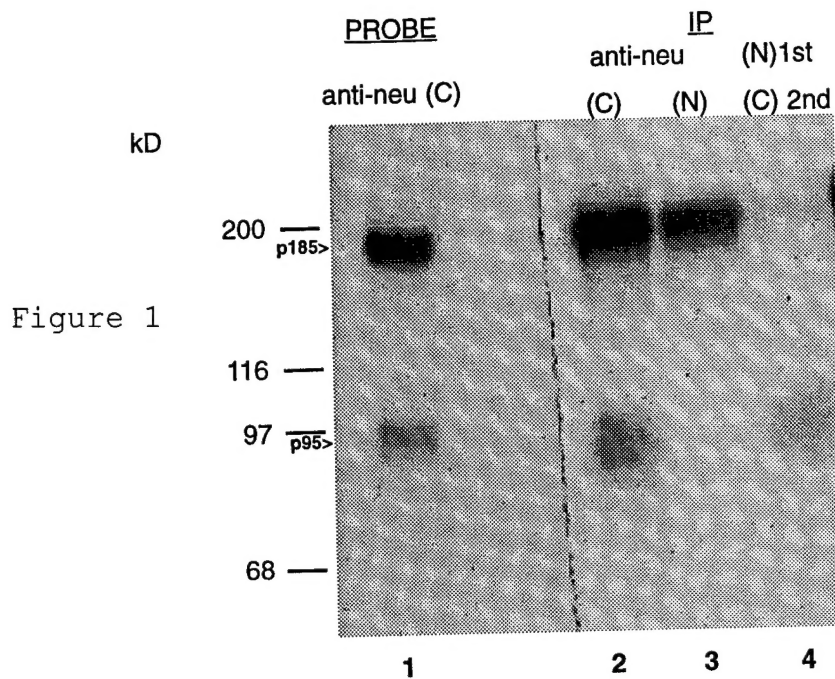


Figure 3

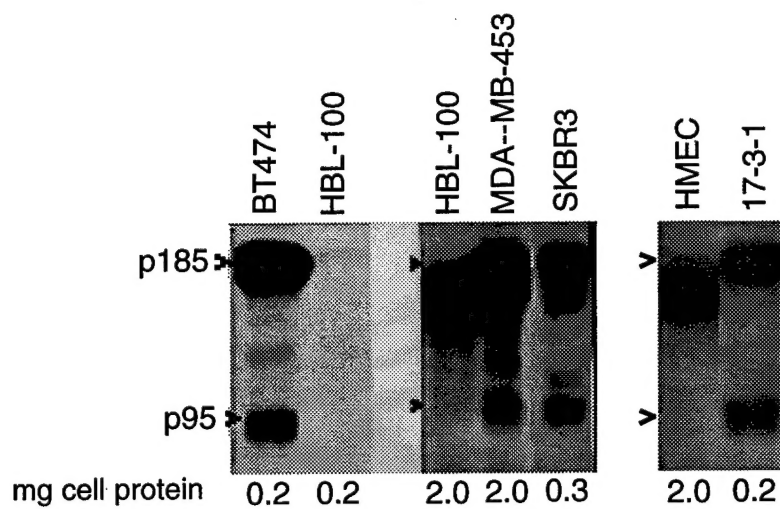


Figure 2

Figure 4

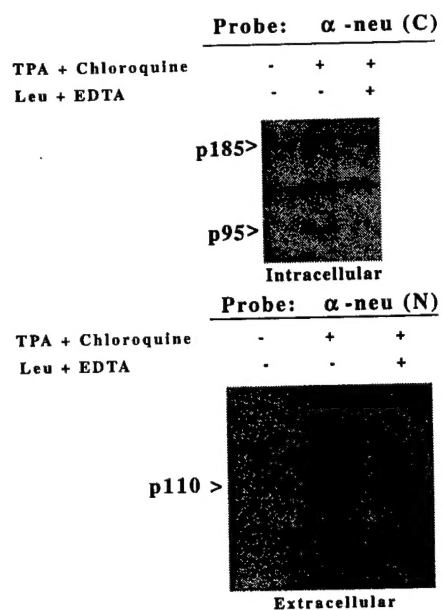


Figure 5

